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Comparison of real-time PCR methods for the detection of *Naegleria fowleri* in surface water and sediment

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Abstract

Naegleria fowleri is a thermophilic free-living ameba found in freshwater environments worldwide. It is the cause of a rare but potentially fatal disease in humans known as primary amebic meningoencephalitis. Established *N. fowleri* detection methods rely on conventional culture techniques and morphological examination followed by molecular testing. Multiple alternative real-time PCR assays have been published for rapid detection of *Naegleria* spp. and *N. fowleri*. Four such assays were evaluated for the detection of *N. fowleri* from surface water and sediment. The assays were compared for thermodynamic stability, analytical sensitivity and specificity, detection limits, humic acid inhibition effects, and performance with seeded environmental matrices. Twenty-one ameba isolates were included in the DNA panel used for analytical sensitivity and specificity analyses. *N. fowleri* genotypes I and III were used for method performance testing. Two of the real-time PCR assays were determined to yield similar performance data for specificity and sensitivity for detecting *N. fowleri* in environmental matrices.

Keywords

Naegleria fowleri; Real-time PCR; Environmental detection; Method comparison

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Introduction

Naegleria fowleri is a thermophilic free-living ameba that can tolerate temperatures of up to 46 °C and is found in freshwater and soil worldwide (Griffin 1972; De Jonckheere 2002; Visvesvara et al. 2007). It has three morphological forms: trophozoite, flagellate, and cyst. The trophozoite form is responsible for infection (Marciano-Cabral 1988). *N. fowleri*, the only *Naegleria* spp. known to cause disease in humans, is the pathogen responsible for primary amebic meningoencephalitis (PAM), which typically results in death within 3–7 days after onset of symptoms (Visvesvara et al. 2007; Yoder et al. 2010; De Jonckheere 2011). For infection to occur, *N. fowleri* must enter the nasal cavity and then migrate to the brain where the organism provokes inflammation and tissue destruction (John 1982; Visvesvara et al. 2007). The clinical presentation of PAM may include headache, fever, nausea, vomiting, and neck stiffness with later progression to loss of balance, seizures, coma, hallucinations, and death (Marciano-Cabral and Cabral 2007). Most infections have been attributed to swimming in bodies of freshwater during warm months. Other water sources associated with exposure in the USA include geothermally heated water, improperly chlorinated swimming pools, recreational water, and nasal rinsing with tap water (Yoder et al. 2010, 2012).

Established detection methods for *N. fowleri* rely on conventional culture techniques and morphological examination, followed by molecular testing. Although traditional methods are effective, they can be time-consuming and often require a combination of techniques in order to be highly specific. Molecular analytical methods are the most feasible approach for confirming the presence of *N. fowleri* in a sample. Multiple molecular methods have been reported for the detection and/ or quantification of *Naegleria* spp. or, specifically, *N. fowleri* (Réveiller et al. 2002; Marciano-Cabral et al. 2003; Behets et al. 2006, 2007; Qvarnstrom et al. 2006; Robinson et al. 2006; Puzon et al. 2009; Lares-Villa and Hernández-Peña 2010; Ahmad et al. 2011). These assays generally target the 5.8S rDNA gene, ITS1 or ITS2 regions. Although these assays have been reported to successfully detect *Naegleria*, many have been developed for clinical diagnostics and have not been evaluated for detection of *N. fowleri* in environmental samples. In this study, we compared four real-time PCR assays (Qvarnstrom et al. 2006; Robinson et al. 2006; Puzon et al. 2009; Mull et al. 2013) for the detection of *N. fowleri* using the following parameters: (1) thermodynamic stability, (2) assay specificity and sensitivity, (3) limit of detection (LOD), (4) assay inhibition associated with humic acid, and (5) assay performance with environmental samples.

Materials and methods

Sources of amebas

Isolates included in this study represented four genotypes of *N. fowleri*, nonpathogenic *Naegleria* strains, and other ameba typically found in freshwater environments (Table 1). Ameba isolates were obtained from the American Type Culture Collection (ATCC) and the laboratory of Dr. Govinda Visvesvara (CDC). Isolates of *N. fowleri* genotypes I, II, III, and IV originated from patient cerebrospinal fluid samples submitted to the CDC for diagnostic purposes. Of the eight identified genotypes of *N. fowleri*, three have been found in the USA (genotypes I, II, and III), one of which has only been reported in California (genotype I) (De

Jonckheere 2011). All ameba stocks were cultured on non-nutrient agar (NNA) plates inoculated with *Escherichia coli* (ATCC Strain #11775, Manassas, VA) and incubated at 37 °C for 48 h. Ameba cultures were harvested from NNA plates by adding 2-mL WB saline (Visvesvara and Balamuth 1975) and scraping the surface of the agar with a sterile scraper. Approximately 1.4 mL of the suspension was removed and added to a sterile microcentrifuge tube, 700 μL of which was used for DNA extraction and the remainder used for subculturing. Cultured ameba concentrations were determined by counts on a Thoma hemacytometer, using ×400 total magnification on a standard light microscope.

Real-time PCR

DNA was extracted from ameba stock cultures using a lysis buffer (Phthisis Diagnostics/ Microbiologics, catalog #E003-100) containing 4.5 M guanidinium isothiocyanate (Hill et al. 2007, 2010). Briefly, 700 μL of lysis buffer was added to 700 μL of harvested ameba in a 2-mL screw-cap polypropylene tube (National Scientific Supply, Claremont, CA) that contained 200 mg each of 0.2- and 0.5-mm acid-washed ZrOx beads. Samples were placed in a Mini-Bead-Beater-8 (Biospec, Bartlesville, OK) and were shaken for 1 min at maximum speed. After bead-beating, samples were centrifuged at 10,000×*g* for 30 s. Following centrifugation, samples were added to a nucleic acid-binding silica column (Omega Biotek, Norcross, GA) and were washed with equal amounts of 100 and 70 % ethanol. Nucleic acid was eluted in 80-μL Tris-EDTA buffer (TE) (Life Technologies, Carlsbad, CA).

Each real-time PCR assay investigated in this study targeted a different *N. fowleri* genetic sequence: assay A, 5.8S rRNA gene and ITS region (Mull et al. 2013); assay B, 18S rRNA gene (Qvarnstrom et al. 2006); assay C, 5.8S rRNA gene (Robinson et al. 2006); and assay D, ITS region (Puzon et al. 2009). For all assays, the initial denaturation conditions were standardized to 95 °C for 10 min, according to the specifications of the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA) used for all reactions. Each PCR reaction was performed with 5-μL DNA template in a total reaction volume of 50 μL. The number of real-time PCR cycles was set at 45 for each assay; the cutoff for classifying a reaction as positive was established at a cycle threshold (C_T) value of 42. Cycling conditions for assay A, a TaqMan assay, were 95 °C denaturation for 15 s, followed by annealing and fluorescence acquisition at 63 °C for 33 s (Mull et al. 2013). In addition, PCR facilitators were used in assay A as described in the literature (Mull et al. 2013). The specific PCR facilitators used in assay Awere gene protein 32 (gp32) and bovine serum albumin (BSA). Both reagents have been shown to be useful for reducing PCR inhibition (Hill et al. 2010). The conditions for assay B, another TaqMan assay, were 95 °C denaturation for 15 s, followed by annealing and fluorescence acquisition at 63 °C for 60 s (Qvarnstrom et al. 2006). Cycling conditions for assay C, an intercalating dye assay using SYTO9, were 94 °C denaturation for 20 s, annealing at 50 °C for 20 s, extension at 72 °C for 20 s, and fluorescence acquisition at 80 °C for 6 s (Robinson et al. 2006). A melt curve was obtained after 45 PCR cycles by increasing reaction temperature from 75 to 95 °C in steps of 0.5 °C every 20 s. For assay D (also a SYTO9 assay), the time for acquisition of fluorescence data was changed from 1 to 6 s due to a minimum time limit of 6 s for any PCR step required by the Bio-Rad iQ5 thermal cycler used in this study (BioRad, Hercules, CA). Cycling conditions for assay D were 95 °C denaturation for 30 s, annealing at 52 °C for 30 s,

extension at 72 °C for 45 s, and fluorescence acquisition at 80 °C for 6 s (Puzon et al. 2009). A melt curve was obtained after 45 PCR cycles by increasing reaction temperature from 75 to 95 °C in steps of 0.2 °C every 10 s. A no template control (NTC), a positive control (*N. fowleri* genotype I), and a negative control (nuclease-free water) were included in each real-time PCR run.

Thermodynamic stability, assay sensitivity, and specificity

N. fowleri genotype I (CDC:V212) DNA was used for thermodynamic stability testing using the gradient option on the Bio-Rad iQ5 thermal cycler. Assays A and B were analyzed using a temperature gradient of 58 to 65.8 °C whereas assays C and D were analyzed with a lower gradient of 48 to 58 °C. Temperature gradient choice was based on the optimal annealing temperatures reported for each assay (Qvarnstrom et al. 2006; Mull et al. 2013; Puzon et al. 2009; Robinson et al. 2006). The highest annealing temperature for which there was no appreciable change in C_T value among samples in a given assay (taken as ≤3 C_T values) was considered optimal. The analytical sensitivity and specificity of the four assays were determined using a panel of DNA that included the 21 isolates listed in Table 1. The DNA amount from each isolate culture used in specificity testing was equivalent to 200 amebas/μL (1000 amebas per real-time PCR reaction). The assay that demonstrated the lowest assay sensitivity was removed from further performance experiments in which standard curves, LODs, inhibition, and performance with environmental samples were investigated.

Standard curves and limits of detection

Standard curves for the three remaining assays were determined in triplicate with DNA extracted from stocks of *N. fowleri* genotypes I and III, which contained a known number of ameba trophozoites serially diluted in TE buffer. These genotypes were chosen because they represent two of the three genotypes that cause PAM within the USA (De Jonckheere 2002). Using linear regression, the best fit C_T value was calculated as a function of the log of the ameba titer per reaction to produce a standard curve. Based on the standard curve data for *N. fowleri* genotypes I and III, the PCR efficiency of each assay was calculated using the following equation:

$$\text{PCR efficiency}(\%) = -1 + 10^{(-1/\text{Slope})}.$$

In conjunction with each standard curve, 1:2 dilutions were made of the last DNA concentration in which all three real-time PCR reactions were positive. These dilutions were analyzed in five replicate reactions to estimate the LOD, which represented the DNA template amount corresponding to a detection rate of ≥80 %.

The assay that exhibited a lower PCR efficiency and higher LOD was removed from further experiments in which inhibition and performance with environmental samples were investigated.

Humic acid inhibition

To evaluate potential inhibitory effects of humic acid on the two remaining assays, varying concentrations (0, 5, 10, 20, 30 ng/μL) of Suwanee River Humic Acid Standard II (Cat # 2S101H, International Humic Substances Society, St. Paul, MN) were added to the real-time PCR reaction mixtures carried out in triplicate, each containing the same amount of *N. fowleri* DNA (~100 amebas/reaction, CDC:V212). C_T values were plotted as a function of humic acid concentration to determine the relationship between these two variables.

Performance with sediment and surface water samples

The performance of the two remaining assays was analyzed using seeded surface water and sediment samples from Murphey Candler Lake in Atlanta, Georgia. Additional sediment was collected from Lake Alice in Gainesville, Florida, in order to compare assay performance for different types of sediment. Six 1-L surface water, six 1-L Georgia sediment, and six 1-L Florida sediment samples were collected. One triplicate set of surface water and sediment samples was seeded with approximately 83*N. fowleri* amebas (approximately equal amounts of trophozoites and cysts) whereas another set was seeded with approximately 156*N. fowleri* amebas (approximately equal amounts of trophozoites and cysts) (CDC:V212, genotype I) for final concentrations of 83 amebas/L and 156 amebas/L, respectively. After seeding, surface water samples were centrifuged for 15 min, 1500×*g* to pellet the *N. fowleri* trophozoites and cysts. Sediment samples were washed after seeding with 1 L of WB saline. The resulting supernatant was subsequently centrifuged and processed using the same procedures described for water samples. The pellets containing *N. fowleri* trophozoites and cysts were then resuspended. Resuspended soil pellets ranged from 9 to 27 mL whereas resuspended surface water pellets ranged from 1 to 15 mL.

A volume of 700 μL from each sample pellet was analyzed by PCR without using immunomagnetic separation (IMS). The remaining sample pellet volume was processed prior to PCR analysis using a previously reported IMS procedure (Mull et al. 2013). Nucleic acid from surface water and sediment was extracted using the same protocol (Hill et al. 2010) for both non-IMS and IMS processed samples. The two remaining real-time PCR assays were performed in triplicate using DNA template volumes of 5 μL and a lower volume of 2 μL to account for potential assay inhibition related to the environmental samples.

Statistical analysis

Kruskal-Wallis equality of populations rank test was used to test for differences between the assays for the following parameters: limit of detection, PCR efficiency, overall ΔC_T with increasing humic acid concentration (slope), and ΔC_T for each humic acid concentration level (relative to 0 ng/μL) for assays A and B at a significance value of p=0.05. Statistical analyses were stratified by *N. fowleri* genotype for the limit of detection analyses. Fisher's exact test was used to determine whether the assays performed differently based on results for seeded environmental samples at a significance level of p= 0.05. Analyses were performed in STATA Version 10.1 (College Station, TX).

Results

Thermostability, assay sensitivity, and specificity

Assays A and B yielded consistent C_T values up to an annealing temperature of ~63 °C (Fig. 1), whereas assays C and D did not yield C_T values when annealing temperatures were above 53 and 60 °C, respectively. While the relatively lower thermodynamic stability of assays C and D was expected based on the T_m values of their primers, the gradient PCR data shown in Fig. 1 confirmed that assays A and B can yield consistent C_T values at higher annealing temperatures. In general, real-time PCR assays that can be executed with higher annealing temperatures may result in higher specificity and sensitivity, which is an important consideration for PCR assays used to analyze environmental samples in which *N. fowleri* concentrations may be low and present among a multitude of nontarget amebas. For each assay, the gradient PCR data from the present study identified an optimal annealing temperature that was in agreement with the annealing temperature reported by the authors of each assay (Mull et al. 2013; Qvarnstrom et al. 2006; Robinson et al. 2006; Puzon et al. 2009).

Assays A and B also exhibited similar sensitivity (100 %) and specificity (93 %) (data not shown). Assay B amplified *Willertia magna* at a concentration of 1000 amebas/reaction. However, the strength of the cross-reaction was weak based on comparison of the high C_T value associated with *W. magna* (average C_T=40.44) versus C_T values of ~30 when stocks of *N. fowleri* at 1000 amebas/reaction were amplified. Assay A amplified *Hartmannella vermiformis* at a concentration of 1000 amebas/reaction. The strength of this cross-reaction was also relatively weak (average C_T=40.25) when compared to C_T values of ~30 when stocks of *N. fowleri* at 1000 amebas/reaction were amplified.

For assay C, designed as a genus-specific assay, DNA from 10 of the targeted *Naegleria* spp. strains amplified whereas DNA from 4 of the remaining targeted *Naegleria* spp. strains (including *N. fowleri* genotype IV) did not, resulting in 71 % sensitivity, but 100 % specificity (10/(10+4)*100=71 %; 7/ (7+0)*100=100 %). Melt curves from this assay had 1–2 peaks in the interval 78–82 °C for all species when resolved at 0.5 °C. A third melt curve peak reported by the authors for *N. fowleri* was not observed in the present study. Differences in melt curve profiles may have been due to the use of a different mastermix or the use of a different real-time PCR platform than was used by the authors of assay C (Robinson et al. 2006). As stated by Robinson et al. (2006), the method of analysis of the DNA melting curve data is critical for the resolution of multiple melting domains. However, due to the relatively low percent sensitivity of assay C and the fact that it failed to amplify *N. fowleri* genotype IV, it was not further investigated in this study.

For assay D, DNA from all six *N. fowleri* strains was successfully amplified whereas DNA from other *Naegleria* spp. and freshwater free-living ameba did not amplify, resulting in 100 % sensitivity and 100 % specificity. Melt curves from assay D exhibited two peaks in the interval 78–81.6 °C (peak 1 at 78.3±0.14 °C and peak 2 at 81.0±0.55 °C), which differed from melt curve peak temperatures reported by the authors (peak 1 at 81.3±0.3 °C and peak 2 at 84.2±0.4 °C) (Puzon et al. 2009). However, the temperature differential between peaks

1 and 2 was similar for both studies (2.9 °C reported by the authors versus 2.7 °C in the present study).

Standard curves and limits of detection

Standard curves for assays A, B, and D were plotted as C_T versus the log of ameba titer per reaction (Fig. 2). Assay D had a lower overall PCR efficiency for both genotypes I (82.9 %) and III (71.5 %) versus assay A and assay B. While assay A had the lowest PCR efficiency for genotype I (76.8 %), it had the highest for genotype III (99.4 %). Assay B had more consistent, and overall higher, PCR efficiency for both genotypes I (89.4 %) and genotype III (94.0 %). The difference in mean PCR efficiencies of the two genotypes combined between assay A and D and between assay B and D was found to be significant (p=0.0039 and p=0.0104, respectively). However, the difference in mean PCR efficiencies between assays A and B was not found to be statistically different (p=0.6310).

LOD testing indicated that assays A and B exhibited lower overall assay detection limits than assay D. For assay A, the LOD was determined to be 0.2 amebas/reaction for genotype I and 0.05 amebas/reaction for genotype III. For assay B, the LOD was determined to be 0.1 amebas/reaction for genotype I and 0.2 amebas/reaction for genotype III. For assay D, a similar detection limit was observed for genotype I (0.2 amebas/reaction), but a higher detection limit was observed for genotype III (0.8 amebas/reaction). The detection limits for assay D were significantly different than the detection limits for assays A and B (p=0.0008 for assay A versus assay D; p= 0.0006 for assay B versus assay D) not taking genotype into consideration. When stratifying by genotype I, the LOD for assay D was found to be significantly higher than the LOD for assay B (p=0.0413). However, there was no significant difference between the detection limits of assay Aversus assay D (p=0.1666). When stratifying by genotype III, the LOD for assay D was found to be significantly higher than the LOD for assays A and B (p=0.0071 for assay A versus assay D and p= 0.0076 for assay B versus assay D). There was no significant difference between the detection limits for assays A and B when combining genotypes (p=0.6987) or when stratifying by genotype (p=0.1653 genotype I and p=0.2123 genotype III).

In environmental applications, where target pathogen levels are often very low, sensitivity is an important parameter to consider when selecting a PCR assay. Based on the results of statistical analyses, assay D was removed from further testing due to a significantly lower PCR efficiency and higher LOD for genotype III versus assays A and B.

Humic acid inhibition

Over a range of 0–30 ng/μL of humic acid, assay B demonstrated less susceptibility to inhibition, with the ΔC_T value at 30 ng/μL (versus 0 ng/μL)=7.0 (Fig. 3). When this concentration of humic acid was present in assay A, the ΔC_T value (versus 0 ng/μL) was 11.8. The slope of the C_T value versus humic acid concentration relationship was 0.3995 for assay A and 0.2263 for assay B. The difference in slope was found to be statistically significant (p=0.0495). The ΔC_T value at each humic acid concentration level tested, relative to 0 ng/μL, was found to be statistically different between the two assays (p<0.05 for 5, 10, 20, 30, and 50 ng/μL). Assay B resulted in lower C_T values (Fig. 3).

Performance with sediment and surface water samples

Table 2 summarizes results for experiments where sediment and surface water samples were seeded with *N. fowleri* genotype I and tested using assay A and assay B after sample processing. Overall, 25 of the seeded samples that were analyzed with assay A were positive whereas 29 of the seeded samples that were analyzed with assay B were positive. This difference in detection between the two assays was not statistically significant (p=0.606). For both assays, the IMS procedure was associated with more *N. fowleri* PCR detections, but the association was not both assays, the IMS procedure *N. fowleri* PCR detections, but significant (p=0.228 overall, p=0.621 for assay A, p= 0.337 for assay B). Using a smaller volume of sample (e.g., 2 μL) in the real-time PCR reaction can result in improved nucleic acid amplification when inhibitors are present, but this technique did not appear to improve detection rates for either assay. In addition, there was no detection in the Georgia sediment regardless of real-time PCR assay, amount of amebas seeded, and DNA template volume. *N. fowleri* could be detected in the Florida sediment when processed using IMS for low seed volumes and when processed using either the direct method or IMS for high seed volumes.

Discussion

The results of this study indicated that assays A and B yielded similar performance data. Assays C and D were not assessed to the same extent as assays A and B based on relatively lower performance results for sensitivity, PCR efficiency, and LOD. This decision to focus further study resources on assays A and B was a resource management decision and was not meant to suggest that assays C and D may not be effective for other applications. Assay C (Robinson et al. 2006) was not designed to be specific for *N. fowleri*, but the researchers determined that *Naegleria* species could be differentiated based on unique melt curve profiles. In the current study, we were unable to replicate the melt curve results reported by the authors of assay C (Robinson et al. 2006), thereby making it difficult to distinguish *N. fowleri* from other ameba species. However, it should be noted that the present study was performed using a different mastermix than was used by the authors of assay C and the PCR protocol had to be changed slightly to enable the assay to be performed on a Bio-Rad iQ5 instrument.

In addition to high specificity, the detection limit of an environmental assay should be relatively low in order to have confidence in detecting target organisms that may be present at low concentrations. Because assay D had a relatively higher detection limit for *N. fowleri* genotype III (0.8 amebas/reaction), it was decided to focus further study resources on evaluating assays A and B. However, it should be noted that this assay was developed by an Australian research group and genotype III has not been reported to cause PAM cases in Australia (De Jonckheere 2002).

Assays A and B performed well at each stage of analysis. Both assays had identical percent sensitivity and specificity, thermodynamic stability, and performance with seeded environmental matrices. Assay A had a lower detection limit for *N. fowleri* genotype III whereas assay B had a somewhat lower detection limit for *N. fowleri* genotype I. In terms of humic acid inhibition, assay B was more robust, based on data indicating less impact by humic acid on assay B C_T values. Humic compounds are the most commonly reported group

of inhibitors in environmental samples (Wilson 1997). Assays A and B appeared to perform better when the environmental samples were processed using IMS, but the effect was not statistically significant. IMS has been shown by other researchers to be an effective technique for removing inhibitors and improving PCR performance (Jiang et al. 2005). However, the addition of an additional sample processing step (IMS) is likely to be associated with some loss of target microbes [as indicated by the ~60 % recovery efficiency of *N. fowleri* cysts and ~90 % recovery efficiency of *N. fowleri* trophozoites reported for the IMS procedure by Mull et al. (2013)].

Neither assay A nor assay B was able to detect *N. fowleri* from seeded Georgia sediment samples. A physiochemical analysis of both the Georgia and Florida sediment samples was performed by an external laboratory (Midwest Laboratories, Inc., Omaha, NE). Results of this analysis suggested that the Georgia sediment had characteristics that may have led to increased real-time PCR inhibition, such as a higher percentage of organic matter (1.0 % for Georgia sediment versus 0.3 % for Florida sediment). In addition, based on the physical appearance of the Georgia and Florida sediments and geographic distribution, the two types of sediment belong to different soil orders (Georgia sediment belongs to Ultisol order and Florida sediment belongs to Spodosol order) (Natural Resources Conservation Service 2013). These physiochemical differences between the two types of sediment might explain the inability to detect seeded *N. fowleri* in the Georgia sediment using real-time PCR. This highlights the need for development of sample preparation techniques to remove PCR inhibitors and adaptation of molecular assays to overcome inhibitory effects associated with challenging environmental matrices. Assay A was reported to effectively detect *N. fowleri* in sediment and water samples in a study where IMS and PCR facilitators were used to reduce PCR inhibition (Mull et al. 2013). Assay B was used for the detection and quantification of *N. fowleri* in a Texas lake commonly used for recreational purposes (Painter et al. 2013). Reported successful detection of *N. fowleri* at concentrations as low as 1–2 cells per 100-mL water sample (Painter et al. 2013) supports the use of assay B in environmental studies. For copy number quantification, the authors assumed that there are approximately 10 copies of the target 18S rRNA gene per diploid *N. fowleri* cell (Painter et al. 2013). Based on the present study results and previous studies, both assay A and assay B appear to be similarly effective alternatives for testing water and sediment samples for *N. fowleri*.

Conclusion

N. fowleri continues to be a rare but tragic cause of water-related mortality. Little is known about the ecology of *N. fowleri* and why certain water bodies are associated with cases of PAM. There is concern that global climate change could potentially increase the risk for PAM in new geographical areas because of the higher incidence of *N. fowleri* in warm water bodies and in warmer months. Improving our understanding of the ecological factors that affect the dynamics of this pathogen in surface water and sediment is important for identifying potential risk factors related to PAM infection. Improved analytical methods will facilitate such studies of *N. fowleri* in environmental systems.

The results from this study provide performance characterization data that can be used to select real-time PCR methods for detection of *N. fowleri* in water and sediment samples. The

two molecular methods identified in this study as being similarly effective for environmental analysis represent useful tools for researchers studying the presence and dynamics of *N. fowleri* in environmental systems, although improved sample preparation and amplification techniques are still needed to reduce inhibition and improve real-time PCR effectiveness for detecting this organism in complex environmental matrices such as sediment.

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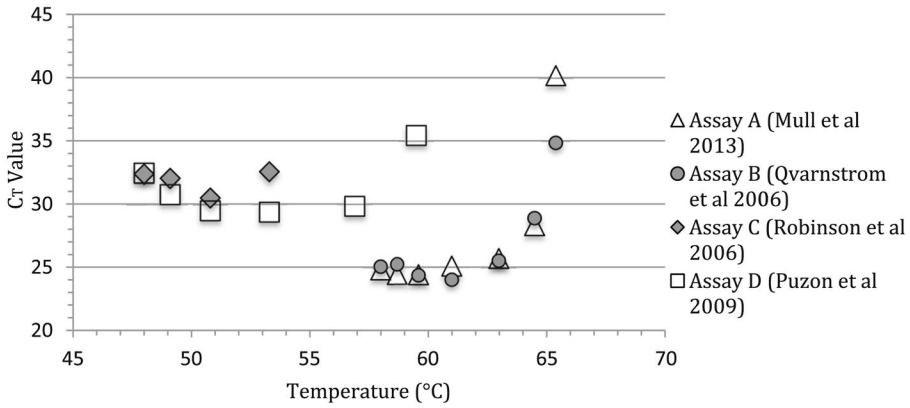

Fig. 1.

Thermodynamic stability of the primers used in assays A, B, C, and D

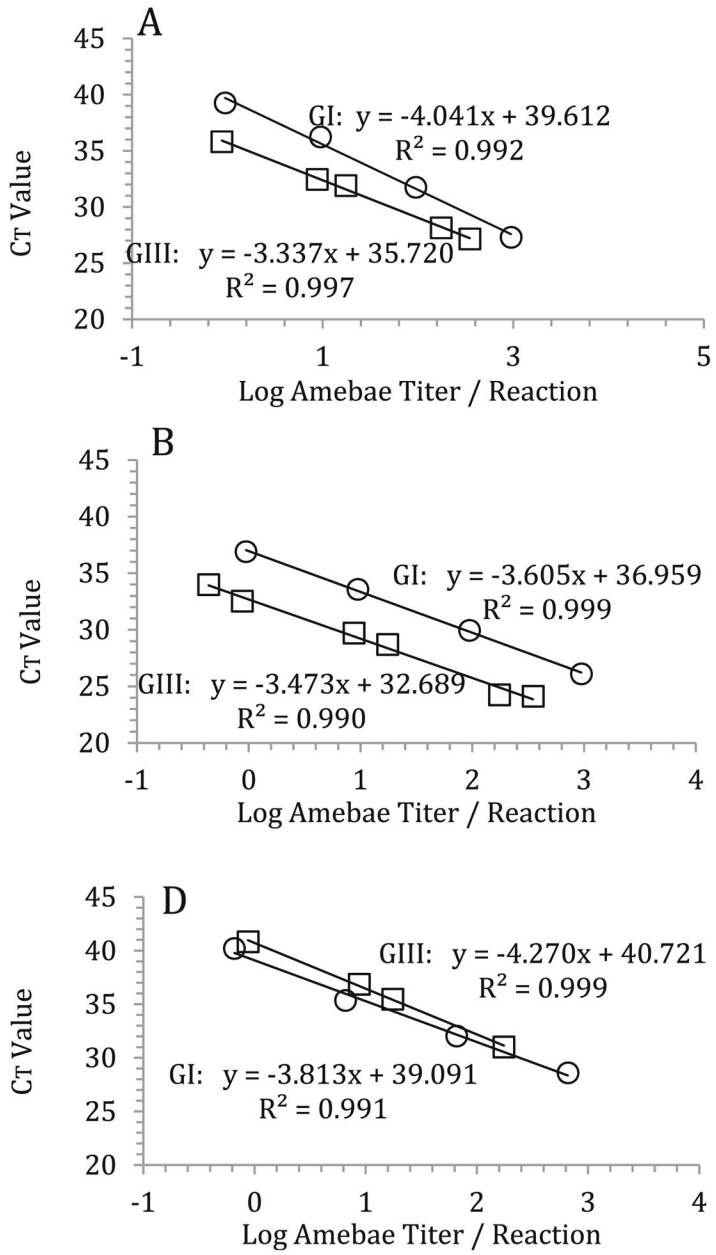

Fig. 2.

Standard curves for assay A (Mull et al. 2013), assay B (Qvarnstrom et al. 2006), and assay D (Puzon et al. 2009) for amplification of *N. fowleri* genotype I (*white circle*) and genotype III (*white square*)

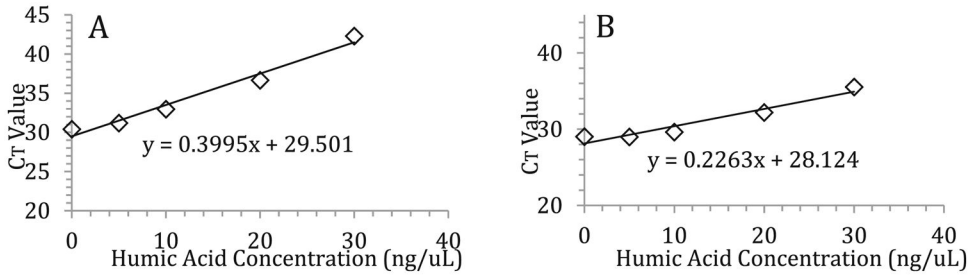

Fig. 3.

CT value as a function of humic acid concentration (ng/μL) for assay A (**a**) (Mull et al. 2013) and assay B (**b**) (Qvarnstrom et al. 2006)

Table 1

Ameba isolates used in this study

Isolate ID	Species	Origin (country/state)
30898	*Acanthamoeba castellanii*	Well water (OH, USA)
50171	*Echinamoeba exundans*	Hot water tank (CA, USA)
50237	*Hartmannella vermiformis*	Hospital Cooling Tower Drain (SD, USA)
30958	*N. australiensis*	Flood Drainage Water (South Australia)
30544	*N. clarki*	Sewage effluent (OH, USA)
PRA-166	*N. dunnebackei*	Livestock water trough (CA, USA)
CDC:V020	*N. fowleri* (genotype I)	CSF (TX, USA)
CDC:V212	*N. fowleri* (genotype I)	CSF (AL, USA)
CDC: V511	*N. fowleri* (genotype I)	CSF (GA, USA)
CAMP	*N. fowleri* (genotype II)	CSF (CA, USA)
CDC: V515	*N. fowleri* (genotype III)	CSF (AZ, USA)
30462	*N. fowleri* (genotype IV)	CSF (Port Pirie, Australia)
30877	*N. gruberi*	Fresh water (AL, USA)
PRA-153	*N. italica*	Fresh water (Pantano Villa, Peru)
30900	*N. jadini*	Swimming Pool (Antwerp, Belgium)
30811	*N. lovaniensis*	Thermally polluted canal (Belgium)
30467	*N. lovaniensis*	Domestic water Supply (Kadina, Australia)
30703	*Tetramitus jugosus*	Stream (Moscow Mountain, ID, USA)
30965	*Vahlkampfia inornata*	Fresh water (WI, USA)
30298	*Vahlkampfia lobospinosa*	Cattle feces (TN, USA)
50036	*Willaertia magna*	Thermally polluted water, nuclear power plant (Belgium)

CSF cerebrospinal fluid

Table 2

Performance with sediment and surface water samples when seeded with *N. fowleri* genotype I (# positive samples/total # seeded samples)

Assay	Matrix	Direct analysis (no IMS)		With IMS	
		5µL DNA	2µL DNA	5µL DNA	2µL DNA
Low seed (~83 amebae)[a]					
A	GA Sediment	0/3	0/3	0/3	0/3
	FL Sediment	0/3	0/3	1/3	1/3
	GA Water	0/3	1/3	1/3	1/3
B	GA Sediment	0/3	0/3	0/3	0/3
	FL Sediment	0/3	0/3	2/3	1/3
	GA Water	2/3	0/3	1/3	1/3
High seed (~156 amebae)[a]					
A	GA Sediment	0/3	0/3	0/3	0/3
	FL Sediment	2/3	2/3	2/3	2/3
	GA Water	3/3	3/3	3/3	3/3
B	GA Sediment	0/3	0/3	0/3	0/3
	FL Sediment	2/3	2/3	3/3	3/3
	GA Water	3/3	3/3	3/3	3/3
Combined detection frequencies		Direct analysis (no IMS)		With IMS	
A	GA Sediment	0/12		0/12	
	FL Sediment	4/12		6/12	
	GA Water	7/12		8/12	
B	GA Sediment	0/12		0/12	
	FL Sediment	4/12		9/12	
	GA Water	8/12		8/12	

[a]Genotype I (CDC:V212)

GA Georgia, *FL* Florida